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Inhibition of Established Subcutaneous and Metastatic Murine Tumors by Intramuscular Electroporation of the Interleukin-12 Gene

Shan-Chih Lee[a,b] Chang-Jer Wu[b] Pin-Yi Wu[b] Yi-Ling Huang[b]
Cheng-Wen Wu[a] Mi-Hua Tao[b]

[a]National Health Research Institutes, Taipei, [b]Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan, ROC

Key Words
In vivo electroporation · Interleukin-12 · Cytokine gene therapy · Antitumor effect · 38C13 B-cell lymphoma · CT-26 colon adenocarcinoma · B16F1 melanoma

Abstract
In vivo electroporation (EP) of the murine interleukin-12 (IL-12) gene in an expression plasmid (pIL-12) was evaluated for antitumor activity. EP transfer of pIL-12 into mouse quadriceps muscles elicited significant levels of serum IL-12 and interferon-γ. Intramuscular EP of pIL-12 resulted in complete regression or substantial inhibition of 38C13 B-cell lymphoma, whereas pIL-12 delivered by gene gun or intramuscular injection without EP showed little therapeutic effect. Impressive antitumor activity by intramuscular EP was also demonstrated in animals with advanced malignant disease. At day 14 after 38C13 tumor inoculation, all animals were found to carry large tumors and to have metastases; without treatment, most died within a week. A single intramuscular EP of pIL-12 resulted in regression of 50% of large subcutaneous tumors and significantly prolonged the lifespan of these animals. Moreover, animals that were previously cured of 38C13 tumors by in vivo EP treatment significantly suppressed tumor growth when challenged 60 days later. In vivo EP of the IL-12 gene was also effective in suppressing subcutaneous and lung metastatic tumors of CT-26 colon adenocarcinoma and B16F1 melanoma cells. Together, these results show that intramuscular electrotransfer of the IL-12 gene may represent a simple and effective strategy for cancer treatment.

Introduction

Many cytokines, either administered systemically or expressed as transgenes by tumor cells, have been intensively investigated as potential anticancer agents. Among the cytokines evaluated, interleukin-12 (IL-12) has been shown to confer potent antitumor activities. IL-12 is a heterodimeric cytokine produced primarily by activated antigen-presenting cells and which mediates a broad range of effects on both innate and acquired immunity [16]. It has been well documented that IL-12 can augment the cytotoxic activities of natural killer (NK) cells and cytotoxic T lymphocytes (CTLs), facilitate type 1 T helper (Th) cell development, and regulate production of many cytokines, particularly interferon-γ (IFN-γ) production from NK and T cells [8, 18]. IL-12 also possesses IFN-γ- and IFN-inducible protein 10-dependent antiangiogenic activity [13]. These diverse biological functions make IL-12 a potent therapeutic agent for malignant diseases [44, 49]. Administration of recombinant IL-12 locally or systemically has been reported to induce potent antitumor

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Mi-Hua Tao, PhD
Institute of Biomedical Sciences
Academia Sinica, 128 Yen-Jiou Yuan Rd., Sec. 2
Nankang, Taipei 11529, Taiwan (ROC)
Tel. +886 2 26523078, Fax +886 2 27829142, E-Mail bmtao@ibms.sinica.edu.tw

activity in a variety of murine tumor models, causing regression of established tumors [5, 34, 53] and inhibiting formation of both experimental metastases [5, 34] and spontaneous metastases [3]. However, in these studies, repeated delivery of recombinant IL-12 on a daily basis was required to achieve the maximal therapeutic activity, which was also usually associated with dose-dependent toxicity [11, 17]. Alternatively, recombinant viruses, including retroviruses [55], pox viruses [29], and adenoviruses [4, 9], have been used to deliver IL-12 systemically or by local injection of high titers of a virus into the tumor mass. Modification of fibroblasts [45, 56], tumor cells [46], or dendritic cells [37] by viral or nonviral vectors has also been used to deliver IL-12. These alternative approaches for IL-12 delivery have various limitations, such as the induction of host antivector cellular immunity in the adenovirus system [22], potential integrational mutagenesis in the retroviral system [31], and a relatively low transfection efficiency of nonviral plasmid DNA, even when delivered in complexes with cationic liposomes [24].

Electroporation (EP) has been widely used to introduce exogenous molecules, including DNA, into cultured cells [35, 54]. This system provides much higher transfection efficiencies compared with other nonviral transfer systems. EP has also been used to transfer chemotherapeutic agents into tumors in vivo, a process known as electrochemotherapy. The combination of a local injection of an anticancer drug, such as bleomycin, and in vivo EP has been shown to be an effective anticancer treatment in a variety of animal models for different types of cancers [14, 21, 33]. Moreover, electrochemotherapy for human malignant tumors has achieved significant (33–96%) complete response rates in several clinical trials [21]. Recently, in vivo EP was shown to be effective in introducing reporter genes into a variety of organs and tissues, including mouse muscles [1], mouse skin [48], mouse myeloma [43], chicken embryos [32], rat liver [20], rat brain [36], and rat corneal endothelium [39]. This approach has also been successfully used in animal models for the production of functional proteins, such as erythropoietin [28] and interleukin-5 [1], from transfected muscle tissues. These studies clearly demonstrate that gene transfer into muscles by in vivo EP is more efficient for producing sustained serum levels of therapeutic proteins than is a simple intramuscular DNA injection.

In the present study, we investigated whether in vivo EP can be applied in cytokine gene therapy for treating malignant diseases. A plasmid vector (pIL-12) encoding the p35 and p40 subunits of murine IL-12 was electrotransferred into muscle tissues to treat a variety of subcutaneous or metastatic murine tumors, including 38C13 B-cell lymphomas, CT-26 colon adenocarcinomas, and B16F1 melanomas. We also compared the antitumor activities of in vivo EP with other nonviral gene delivery methods (intramuscular and gene gun delivery).

Materials and Methods

Mice

Female C3H/HeN mice, 10 weeks old, were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). Female BALB/c and C57BL/6 mice, 8 to 10 weeks old, were obtained from the Laboratory Animal Facility, Institute of Biomedical Sciences, Academia Sinica (Taipei, Taiwan). Animal care was provided in accordance with the guidelines approved by the Animal Committee of the Institute of Biomedical Sciences, Academia Sinica, Taiwan.

Cell Lines

38C13 murine B-cell lymphoma is a carcinogen (7,12-dimethylbenz(a)anthracene)-induced tumor originally produced in a T-cell-depleted C3H/eB mouse [2]. CT-26 is a colon adenocarcinoma cell line derived from BALB/c mice treated with N-nitroso-N-methylurethane [12]. B16F1 is a metastasizing subline of the B16 melanoma that arose spontaneously and is syngeneic with C57BL/6 mice [47]. Cell lines were maintained in RPMI-1640, 10% heat-inactivated fetal calf serum, 2 m*M* *L*-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (all from Sigma Chemical, St. Louis, Mo., USA) at 37 °C with 5% CO_2 in a humidified incubator. 38C13 cells were grown in the above medium supplemented with 50 μ*M* 2-mercaptoethanol.

Plasmids and DNA Preparation

The pIL-12 plasmid that produces biologically active murine IL-12 was described previously [10, 25]. It contains the p35 and p40 coding sequences of murine IL-12 under the control of discrete cytomegalovirus promoters. Plasmid pcDNA3 containing the cytomegalovirus early promoter/enhancer sequence was used as a control plasmid in this study. Plasmid DNA was purified from transformed *Escherichia coli* strain DH5α using a Qiagen Plasmid Giga Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and was stored at −70 °C as pellets. The DNA was reconstituted in sterile saline at a concentration of 1 mg/ml for experimental use.

Intramuscular DNA Injection and Electroporation

In vivo EP was performed as previously described [1] with modifications. Briefly, mice were anesthetized with acepromazine maleate (Fermenta Animal Health Co., Kansas, Mo., USA). Fifty micrograms of plasmid DNA was injected into the bilateral quadriceps muscles using a disposable insulin syringe with a 27-gauge needle. A total of 100 μg of plasmid DNA in various combinations of pIL-12 and pcDNA3 was injected into each mouse. Immediately after injection, a pair of electrode needles was inserted into the muscle to a depth of 5 mm to encompass the DNA injection sites, and electric pulses were delivered using an electric pulse generator (Electro Square Porator ECM 830; BTX, San Diego, Calif., USA). The shape of the pulse was

a square wave. The electrodes consisted of a pair of gold-plated stainless steel needles 5 mm in length and 0.8 mm in diameter, with a fixed distance between them of 5 mm. Six pulses of 100 V each were administered to each injection site at a rate of 1 pulse/s, with each pulse being 50 ms in duration.

Gene Gun-Mediated in vivo Gene Transfer

Experiments utilized a hand-held, helium-driven Helios gene delivery system (Bio-Rad, Hercules, Calif., USA). Plasmid DNA was precipitated onto gold particles with an average diameter of 1.6 μm. Particles were suspended in a solution of 0.1 mg/ml polyvinyl pyrrolidine in absolute ethanol. This DNA/gold particle preparation was coated onto the inner surface of Tefzel tubing using a tube loader (Bio-Rad). The tubing was cut into 1.77-cm segments to result in delivery of 0.5 mg of gold and 1.25 μg of plasmid DNA per transfection. Mouse skin overlying and surrounding the target tumor was transfected in vivo with a helium gas pulse at 300 psi.

Collection and Processing of Tissues

At various times after DNA transfer, blood samples were collected from the tail veins of mice, or mice were sacrificed and the entire quadriceps muscle was collected for preparation of a tissue extract. The muscles were immersed in a liquid nitrogen bath, then ground into powder using a mortar and pestle. The muscle powder was mixed in phosphate-buffered saline (PBS; 1 ml per muscle) containing Complete™, a proteinase inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany), and sonicated before collecting the supernatant. Protein concentrations of muscle extracts were determined by a bicinchoninic acid-based protein assay (Pierce, Rockford, Ill., USA) and normalized to 3 mg/ml. Aliquots were stored at −20 °C until analyzed.

Cytokine ELISA

The IL-12 and IFN-γ levels in muscle extracts and serum samples were measured by sandwich ELISA kits purchased from R&D Systems (mouse IL-12 p70 DuoSet ELISA kit; Minneapolis, Minn., USA) and PharMingen (San Diego, Calif., USA), respectively. Analyses were conducted according to the supplier's instructions.

Tumor Challenge and Therapy

Exponentially growing tumor cells were harvested and used for induction of subcutaneous tumors or for metastasis only if their viability exceeded 95%, as determined by trypan blue staining. To generate subcutaneous tumors, syngeneic C3H/HeN, BALB/c, and C57BL/6 mice were injected subcutaneously with 1×10^3 38C13, 1×10^5 CT-26, or 2×10^5 B16F1 tumor cells, respectively, in 100 μl of PBS. Treatment was begun 3 days after tumor inoculation by intramuscular delivery of the plasmids pcDNA3 or pIL-12 with or without EP. For 38C13 tumors, mice were also treated with gene gun delivery of pIL-12. Each gene gun treatment consisted of 4 transfections with a total of 5 μg of plasmid DNA per treatment. One transfection was given directly over the tumor site, and 3 additional transfections were evenly spaced around the circumference of the tumor in a triangular pattern as previously described [41]. For some experiments, mice were treated 7 or 14 days after tumor inoculation with in vivo EP delivery of 100 μg of pcDNA3 or pIL-12. Tumor growth was measured 2 to 3 times per week, and the tumor size (in mm^3) was approximated by using the ellipsoidal formula: length (mm) × width (mm) × height (mm) × 0.52 (derived from π/6). The mean volume and SD of each group were calculated. Animals were observed until the subcutaneous tumors measured more than 3,000 mm^3 or until any mouse was observed to be suffering or appeared to be moribund. Animals under these conditions were euthanized humanely according to institutional policy. Sacrifice dates were recorded, and the mean survival of each group was calculated.

In the lung metastasis models, BALB/c and C57BL/6 mice were given 1×10^5 CT-26 cells or 2×10^5 B16F1 cells, respectively, suspended in 0.5 ml of PBS by tail vein injection. Treatment was begun 3 days after tumor inoculation by intramuscular delivery of the plasmids pcDNA3 or pIL-12 with or without EP. Animals were killed on day 21 for enumeration of surface metastases in the lungs under a dissecting microscope.

Statistics

The statistical significance of differential findings between experimental groups of animals was determined using Student's t test. Findings were regarded as significant if two-tailed p values were ≤ 0.05.

Results

IL-12 Expression by in vivo EP

We previously constructed a bicistronic plasmid, pIL-12, containing the p35 and p40 coding sequences of murine IL-12. To evaluate the effect of pIL-12 in cancer gene therapy, we delivered the IL-12 gene by in vivo EP, which has been shown to dramatically increase gene expression in muscle tissues [1]. C3H/HeN mice were injected in the quadriceps muscles with 100 μg of pIL-12 (50 μg per leg), and 1 group of mice was electrostimulated immediately after injection. Mice that received the control plasmid pcDNA3 followed by electrostimulation served as negative controls. The time course of gene expression was determined by following serum IL-12 levels. As shown in figure 1, no serum IL-12 was detectable within the sensitivity limit of our ELISA assay (<10 pg/ml) in mice that received the control plasmid. Mice in the IL-12 gene-treated but unstimulated group also produced no detectable serum IL-12. In contrast, the serum IL-12 level in mice treated with the IL-12 plasmid with EP increased from 150 ± 30 pg/ml on day 1 to a peak level of 1,430 ± 460 pg/ml on day 5 which subsequently decreased to 150 ± 40 pg/ml by day 11. This low but significant level of serum IL-12 persisted for at least 60 days after a single electrotransfer of the IL-12 plasmid (fig. 1).

We also analyzed IL-12 expression in muscle tissue following IL-12 gene treatment. Animals were given an injection of 100, 10, or 1 μg of pIL-12 (half in each quadriceps muscle) with or without EP, and the IL-12 levels in selected muscles were assayed at day 5 postinjection. Figure 2A shows that a low but significant level of IL-12 expression was present in animals treated with 10

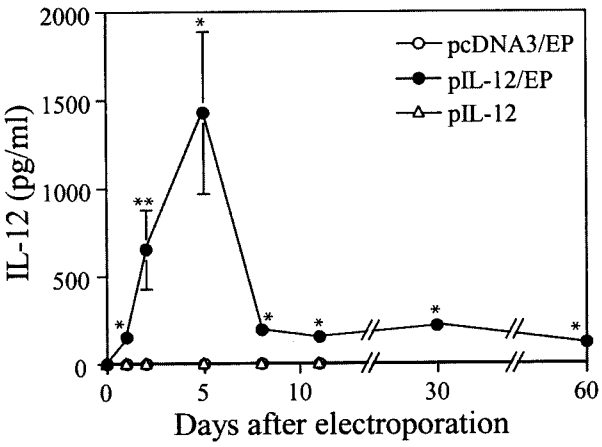

Fig. 1. Serum IL-12 levels after pIL-12 transfer with and without in vivo EP. The bilateral quadriceps muscles of C3H/HeN mice were injected with 100 μg (50 μg in each muscle) of pcDNA3 with EP or pIL-12 plasmid DNA with or without EP. Serum samples were obtained on the indicated days after EP and measured for the presence of IL-12 using a commercial ELISA kit. Each value represents the mean IL-12 concentration ± SD from 5 mice. * $p < 0.01$ and ** $p < 0.05$ vs. the pcDNA3 control. Data are representative results of 2 independent experiments.

or 100 μg of pIL-12 without electrostimulation. Electroporation of the DNA-treated muscle increased the transgene expression at all doses of pIL-12 tested. At a dose of 100 μg, a simple intramuscular injection of pIL-12 produced 80 ± 40 pg/ml of IL-12. This level increased approximately 80-fold to 6,340 ± 1,980 pg/ml after EP.

Taken together, these data indicate that in vivo EP effectively enhanced the efficiency of muscle-targeted IL-12 gene transfer, and moreover, that continuous delivery of low but sustained levels of IL-12 can be achieved by a single plasmid injection using the EP method.

Induction of Serum IFN-γ by EP Delivery of pIL-12

One of the most important properties of IL-12 is its ability to induce the production of IFN-γ from resting and activated T and NK cells. This activity of IL-12 is central to many of the effects seen when IL-12 is administered in vivo, and provides a mechanism whereby IL-12 plays an important role in innate, as well as in adaptive, immunity [16]. To evaluate whether electrotransfer of pIL-12 can express functionally active IL-12 and enhance IFN-γ production in vivo, serum IFN-γ levels of mice receiving various amounts of pIL-12 were measured over time. Mice treated with pIL-12 without EP produced no detectable

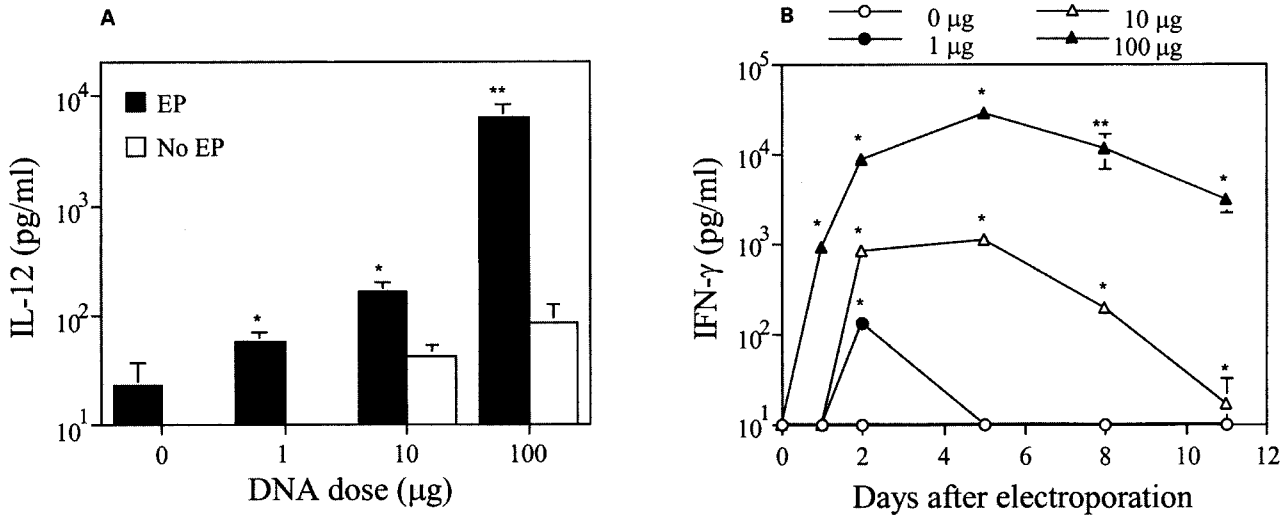

Fig. 2. Effect of pIL-12 dosage on muscle IL-12 expression and serum IFN-γ production. (**A**) Different doses of pIL-12 (0, 1, 10, or 100 μg) were injected into the quadriceps muscles (half in each quadriceps muscle) of C3H/HeN mice with or without EP. Complementary doses of pcDNA3 were administered in such a way that each muscle received a total dose of 50 μg of plasmid DNA. Five days after treatment, animals were sacrificed, and the quadriceps muscles (n = 6) were collected to determine IL-12 expression. Each value represents the mean IL-12 concentration ± SD. (**B**) Mice were treated with different doses (0, 1, 10, or 100 μg) of pIL-12 as shown above and stimulated with electric pulses. Serum samples were collected on the indicated days after EP and measured for the presence of IFN-γ using a commercial ELISA kit. Each value represents the mean IFN-γ concentration ± SD from 5 mice. * $p < 0.01$ and ** $p < 0.05$.

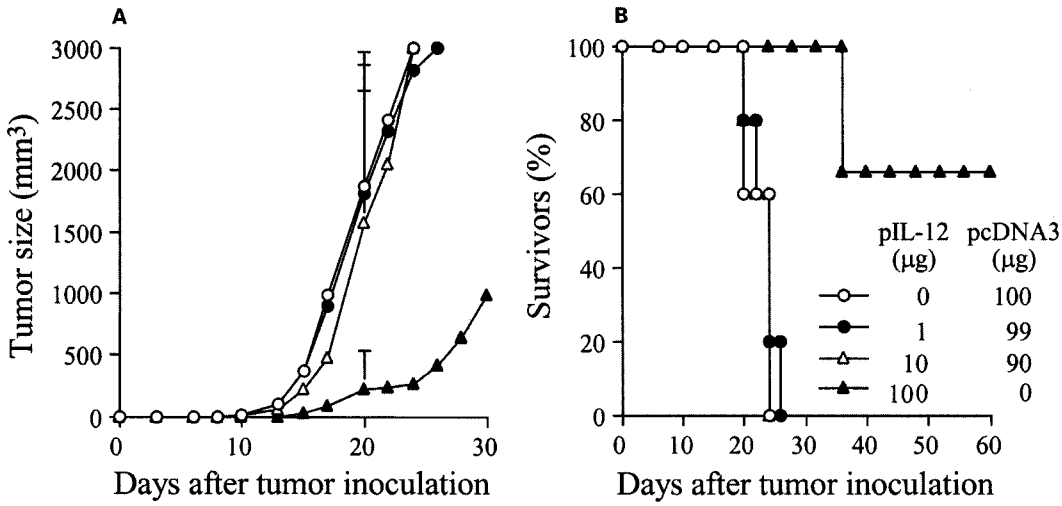

Fig. 3. Suppression of the in vivo growth of 38C13 tumors by intramuscular electrotransfer of pIL-12. Syngeneic C3H/HeN mice (n = 6) were subcutaneously inoculated with 1×10^3 tumor cells on day 0. Three days later, animals were treated with pIL-12 through in vivo EP at doses of 0, 1, 10, or 100 μg. Complementary doses of pcDNA3 were administered in such a way that all animals received a total dose of 100 μg of plasmid DNA (as in fig. 2). Tumor growth was measured 3 times a week. The mean tumor volume (**A**) and the percentage of survivors (**B**) in each group were determined. SDs (bars) are only given at day 20 for clarity. Data are representative results of 2 independent experiments.

serum IFN-γ levels even at the largest dose (100 μg) of DNA tested (data not shown). In contrast, EP stimulated serum IFN-γ production at all doses of DNA. The circulating IFN-γ reached a peak level at 2 to 5 days after IL-12 gene treatment, and had variably decreased in the different groups from 1/10 to 1/100 of the peak value after 11 days (fig. 2B). The serum IFN-γ measured at different time points correlated well with the amount of DNA injected. At day 5 after EP, pIL-12 at doses of 100 and 10 μg produced 28,170 ± 4,860 and 1,110 ± 255 pg/ml of serum IFN-γ, respectively. A lower dose of the plasmid (1 μg of DNA) only produced a low titer (130 ± 25 pg/ml) of IFN-γ at day 2, with the titer decreasing to an undetectable level by day 5. Mice that received an injection of 100 μg of the control pcDNA3 plasmid with EP produced no detectable serum IFN-γ (data not shown). This result demonstrates that EP-mediated IL-12 gene transfer is able to produce a substantial quantity of serum IFN-γ and thus may be capable of systemically stimulating immune cells.

EP-Mediated Transfer of the IL-12 Gene Inhibits Tumor Growth

The antitumor effect of IL-12 gene electrotransfer was evaluated next. Syngeneic C3H/HeN mice were subcutaneously inoculated with 1×10^3 38C13 B cell-lymphoma cells at day 0. Three days later, pIL-12 at doses of 1, 10, or 100 μg was injected into the quadriceps muscles followed immediately by in vivo EP. Complementary doses of pcDNA3 were administered in such a way that all groups of animals received a total dose of 100 μg of plasmid DNA. Mice treated with 100 μg of pcDNA3 alone were included as controls. Tumor volume progression and survival curves are shown in figure 3A and B, respectively. Compared with the control group, intramuscular electrotransfer of 100 μg of pIL-12 resulted in 66% (4 of 6 mice) long-term survivors (>60 days, $p < 0.001$), whereas all animals in the control group had detectable tumors by day 13. In addition, objective tumor growth suppression was observed in tumor-bearing animals in the pIL-12 (100 μg) group (mean survival time 52 ± 5 vs. 22 ± 1 days for the pcDNA3 control group, $p < 0.001$) (fig. 3A). By day 20, the mean tumor volume of the pIL-12 tumor-bearing animals was 220 ± 220 mm^3 as compared with 1,860 ± 490 mm^3 in the pcDNA3 control group. The long-term survivors remained disease-free for an observation period of 120 days and were free of residual or dormant tumor cells as determined by FACS analysis and in vitro culture of spleen and lymph node cells (data not shown). There was no clear dose-effect relationship for the EP-mediated

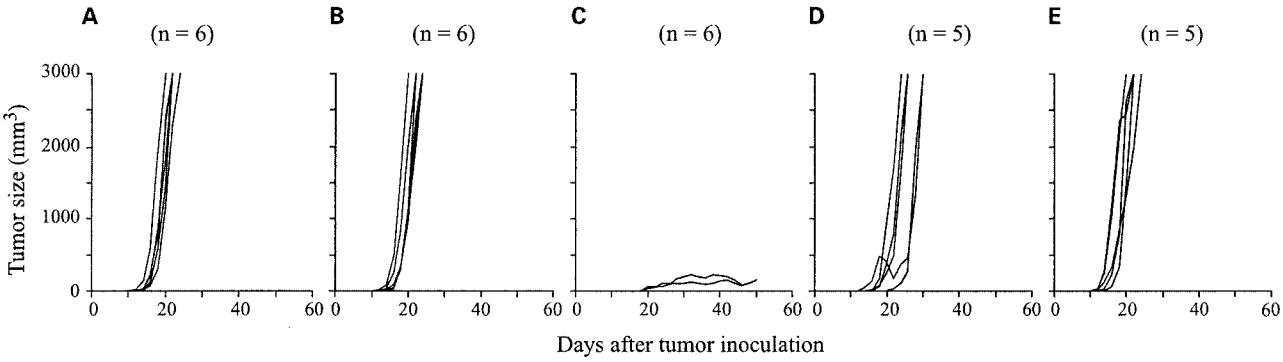

Fig. 4. Comparison of different pIL-12 transfer methods for inhibition of 38C13 tumor growth. C3H/HeN mice (n = 5–6) were subcutaneously inoculated with 1×10^3 tumor cells on day 0. Three days later, animals were randomly divided into 5 groups. Control groups included mice that received no treatment (**A**) or intramuscular electrotransfer of 100 μg of pcDNA3 (**B**). In treatment groups, mice were injected with 100 μg of pIL-12 into the quadriceps muscle with (**C**) or without (**D**) in vivo EP. Mice that received gene gun-mediated pIL-12 transfection were also included (**E**). The tumor volume of individual mice was plotted as a function of time after tumor cell inoculation. Data are representative results of 3 independent experiments.

IL-12 gene therapy. Treatment with 1 or 10 μg of pIL-12 showed no significant inhibition of tumor growth with mean survival times of 23 ± 1 and 24 ± 1 days, respectively, and produced no long-term survivors (fig. 3A, B). Therefore, 100 μg of pIL-12 was used for EP in subsequent experiments.

Comparison of the Ability of Different Transfer Methods to Inhibit Tumor Growth

We next compared the antitumor activities of 3 different nonviral techniques, i.e., direct muscle injection of plasmid DNA, particle-mediated (gene gun) gene transfer, and in vivo EP. Three days after 38C13 tumor inoculation, animals were randomly divided into 5 groups. Mice were injected with 100 μg of pIL-12 into the quadriceps muscles with or without EP or transfected with 5 μg of pIL-12 by gene gun on the skin overlaying and surrounding the target tumor. Mice receiving no treatment or intramuscular electrotransfer with 100 μg of plasmid pcDNA3 were included as controls. As shown in figure 4, treatment with the control pcDNA3 plasmid by in vivo EP did not inhibit 38C13 tumor growth (mean survival time 23 ± 2 vs. 22 ± 1 days for the untreated group, $p > 0.05$). Mice that received a simple intramuscular injection of pIL-12 (fig. 4D) displayed minor tumor suppression with the mean survival time increased by 5 days (to 27 ± 3 days), although the mean survival time did not statistically differ from that of the pcDNA3 control ($p > 0.05$). In contrast, pIL-12 delivered by in vivo EP (fig. 4C) produced 66% (4 of 6 mice) long-term survivors, and resulted in objective tumor growth suppression in the 2 tumor-bearing animals that eventually died due to systemic tumor metastasis (mean survival time of 56 ± 3 days). Interestingly, gene gun delivery of the IL-12 gene at day 3 after tumor inoculation produced no therapeutic antitumor effects (fig. 4E, mean survival time of 22 ± 1 days). This result was in contrast to a previous report that demonstrated a significant antitumor effect after gene gun delivery of the IL-12 gene [41]. To further confirm this result, tumor-bearing mice were treated by daily gene gun transfection of 5 μg of pIL-12 over a period of 5 days. Compared with the control animals, gene gun-mediated multiple transfection of the IL-12 gene led to minor tumor suppression (mean survival time of 29 ± 8 days, $p > 0.05$), but resulted in only 20% (1 of 5 mice) long-term survivors. Taken together, EP-mediated IL-12 gene therapy exhibited the most significant suppression of 38C13 tumor growth as compared with other nonviral gene delivery methods.

Suppression of Established Tumor Growth by Intramuscular Electrotransfer of pIL-12

To further evaluate the antitumor effects of pIL-12 electrotransfer, we performed a more-stringent experiment on animals bearing advanced 38C13 tumors. In

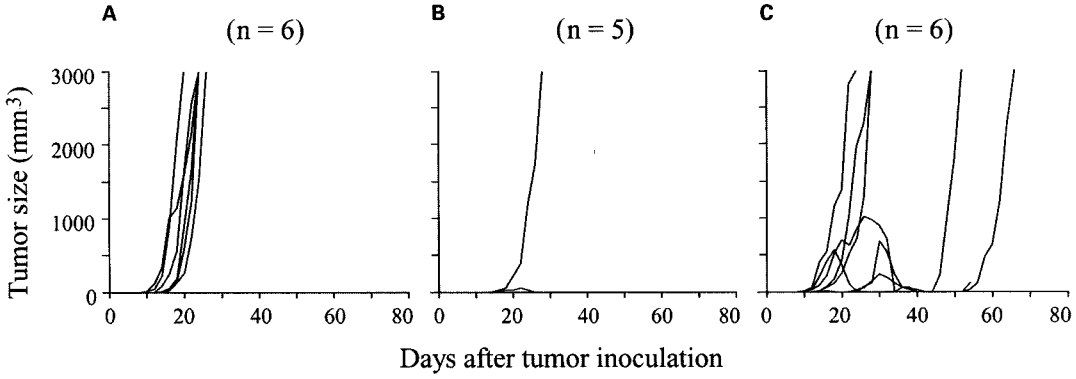

Fig. 5. Treatment of large established 38C13 tumors by intramuscular electrotransfer of pIL-12. C3H/HeN mice (n = 5–6) were subcutaneously inoculated with 1×10^3 tumor cells on day 0. Mice were treated with 100 μg of pIL-12 by in vivo EP on day 7 (**B**) or 14 (**C**). Animals treated with 100 μg of pcDNA3 on day 7 were included as controls (**A**). The tumor volumes of individual mice were plotted as a function of time after tumor cell inoculation. Data are representative results of 3 independent experiments.

untreated mice, 38C13 tumors grew rapidly after subcutaneous injection of 1×10^3 tumor cells, reaching from 2.5 to 3 cm in diameter in 15 to 20 days. In addition, the subcutaneous tumor was capable of spontaneously metastasizing to many lymphoid and nonlymphoid organs, and the mice died around day 22 to 25. To test the therapeutic effect of pIL-12 electrotransfer on large established tumors, C3H/HeN mice were subcutaneously injected with 1×10^3 38C13 tumor cells, and EP transfer of the IL-12 gene was begun on day 7 or 14 after tumor cell inoculation. Electrotransfer of pcDNA3 seven days after tumor cell inoculation served as a control. Compared with the pcDNA3 control group, objective tumor growth suppression was observed in both the day 7- and day 14-treated groups (fig. 5). In the day 7-treated group, complete tumor regression was observed in most animals, with 80% (4 of 5 mice) of the mice remaining tumor-free for more than 80 days (mean survival time 70 ± 23 vs. 25 ± 1 days for the pcDNA3-treated group, $p < 0.01$). Significant results were also observed in the day 14-treated group, at which time all animals bore large tumors (approximately 1,000 mm^3) and had metastases in many organs (up to 12% of total lymph node cells and 3% of spleen cells), and without treatment, many died within a week. A single treatment of pIL-12 by EP on day 14 resulted in regression of 50% (in 3 of 6 mice) of the large subcutaneous tumors and significantly prolonged the lifespan of these animals (mean survival time 42 ± 18 days, $p < 0.05$) (fig. 5C). However, there were no long-term survivors in this group; all animals had died by day 80. These results demonstrate that intramuscular electrotransfer of the IL-12 gene also produced marked therapeutic effects on large established tumors.

On day 60, we rechallenged animals in which 38C13 tumors were eliminated after treatment with pIL-12 by in vivo EP. Age-matched naive C3H/HeN mice that received a subcutaneous injection of 1×10^3 38C13 tumor cells on the same day served as controls. Representative results of 5 independent experiments are shown in figure 6. Significant suppression of tumor growth was observed in the pIL-12-cured group (mean survival time 40 ± 4 vs. 21 ± 1 days for the naive group, $p < 0.0001$). Moreover, 25% (4 of 16 mice) of the pIL-12-cured mice survived and remained tumor-free for an additional 60 days (fig. 6B), whereas all animals (15 of 15 mice) in the naive group succumbed to death by day 24 after tumor cell challenge. These data demonstrate that a single intramuscular electrotransfer of pIL-12 can induce long-term protection in this low-immunogenic tumor model.

Suppression of Tumor Metastasis by Intramuscular Electrotransfer of pIL-12

To further evaluate the antitumor effect of IL-12 electro gene therapy, experiments were extended to 2 additional murine models: CT-26 colon adenocarcinoma and B16F1 melanoma. We first analyzed the therapeutic effect of EP-delivered pIL-12 on subcutaneously injected tumors. Groups of BALB/c and C57BL/6 mice were sub-

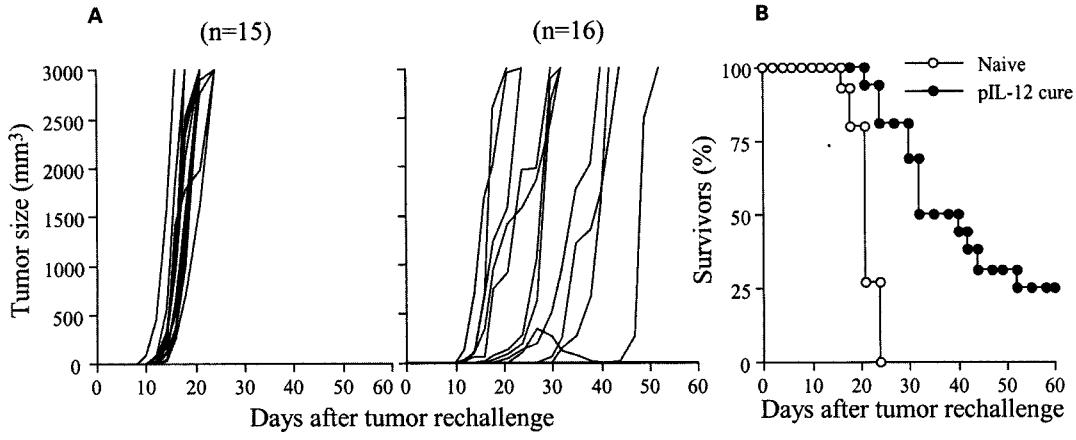

Fig. 6. Long-term protection induced by intramuscular electrotransfer of pIL-12. C3H/HeN mice in which 38C13 tumors had been completely eliminated by IL-12 electro gene therapy were rechallenged by a subcutaneous injection of 1×10^3 38C13 cells on day 60 after treatment. Age-matched naive C3H/HeN mice treated with the same amount of 38C13 cells were included as controls. The tumor volume of individual mice (**A**) and the percentage of survivors (**B**) in each group were determined. Data summarize the results of 5 independent experiments.

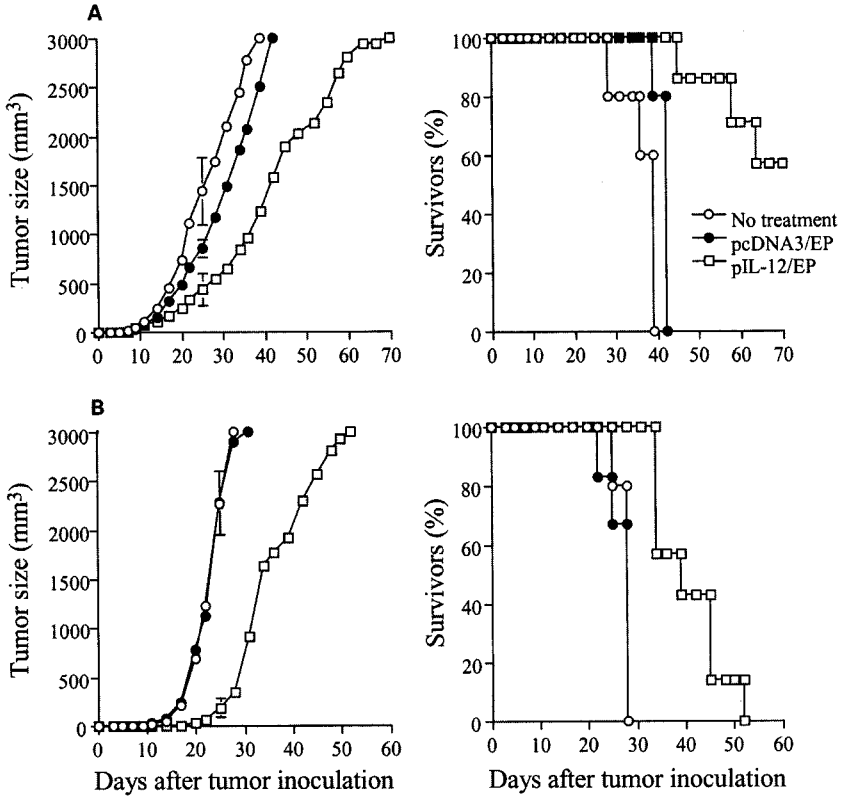

Fig. 7. Suppression of the in vivo growth of CT-26 and B16F1 tumors by intramuscular electrotransfer of pIL-12. BALB/c mice (n = 7) were subcutaneously inoculated with 1×10^5 CT-26 cells (**A**) and C57BL/6 mice (n = 5) with 2×10^5 B16F1 cells (**B**). Three days later, animals were either untreated or treated by intramuscular injection of 100 μg of pIL-12 or pcDNA3 followed by EP. Tumor growth was measured 3 times a week. The mean tumor volume (**A** and **B**, left) and the percentage of survivors (**A** and **B**, right) in each group were determined. SDs (bars) are only given for day 25 for clarity. Data are representative results of 2 independent experiments.

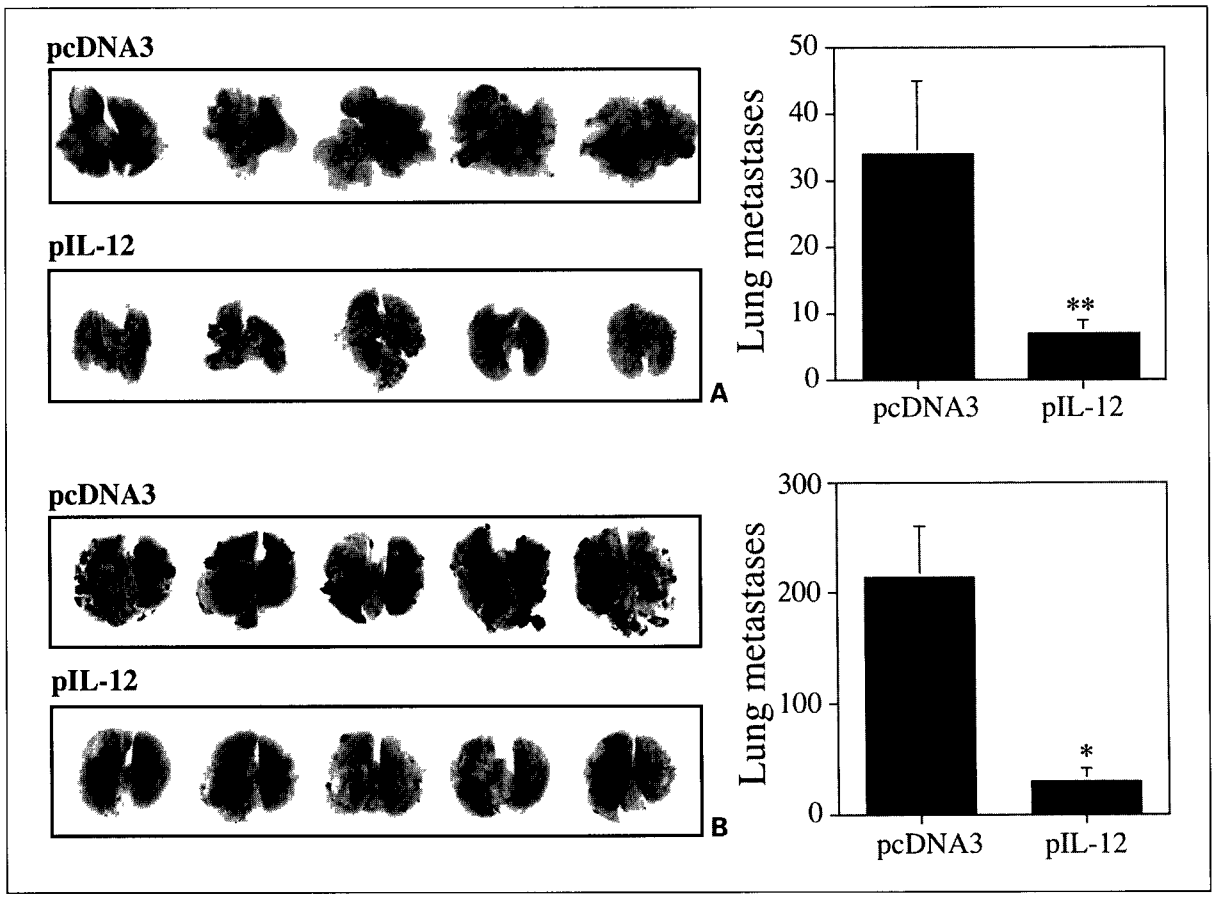

Fig. 8. Inhibition of lung metastases by intramuscular electrotransfer of pIL-12. Experimental lung metastases were induced by intravenous injection of 2×10^5 B16F1 cells into C57BL/6 mice (**A**) or 1×10^5 CT-26 cells into BALB/c mice (**B**). One hundred micrograms of pcDNA3 or pIL-12 was electrotransferred into mice on day 3. Animals were killed on day 21, and tumor nodules in the lung were counted to measure the metastatic load. Data are presented as the mean ± SD of 5 mice per group. Photographs of representative lungs from mice in the control group (**A** and **B**, left top) and the pIL-12/EP-treated group (**A** and **B**, left bottom) are shown. The antimetastatic experiments were repeated 2 times with the CT-26 tumor model and 3 times with the B16F1 model, and similar results were obtained in each experiment. * $p < 0.01$ and ** $p < 0.05$.

cutaneously inoculated with 1×10^5 CT-26 or 2×10^5 B16F1 cells, respectively, and 100 μg of pIL-12 or pcDNA3 plasmid was delivered into the quadriceps muscles with EP 3 days following tumor inoculation. Untreated mice served as controls. As summarized in figure 7, a single intramuscular electrotransfer of 100 μg of pIL-12 resulted in complete tumor regression or suppression of tumor growth in these 2 tumor models. In mice bearing CT-26 tumors, complete tumor regression was achieved in 57% of the tested animals with 4 of 7 mice surviving and remaining tumor-free for more than 70 days. In addition, objective tumor growth suppression was observed in tumor-bearing animals in the pIL-12/EP group (mean survival time 63 ± 4 vs. 41 ± 1 days for the pcDNA3 control group, $p < 0.001$) (fig. 7A). In mice bearing B16F1 melanomas, significant suppression of tumor growth was also observed in the pIL-12/EP-treated group (mean survival time 40 ± 3 vs. 27 ± 1 days for the pcDNA3 control group, $p < 0.001$) (fig. 7B). However, suppression of B16F1 tumor growth was transient, and all animals had eventually died by day 50 from progressing tumors.

To evaluate the antimetastatic effect of pIL-12 electro gene therapy, pulmonary metastases were induced by intravenous injection of 1×10^5 CT-26 cells into BALB/c mice or 2×10^5 B16F1 cells into C57BL/6 mice. After 3 days, microscopically established metastases were present throughout the lung tissue. Grossly visible metastases

were detectable on the surface of the lungs 21 days after tumor cell injection (experimental observation, data not shown). A single intramuscular electrotransfer of 100 μg of pIL-12 on day 3 markedly reduced the macroscopic metastatic lung foci in both the B16F1 and CT-26 tumor models (fig. 8). In the B16F1 tumor model, mice that received pIL-12 by in vivo EP had a mean number of metastatic foci of 29 ± 12 compared with 214 ± 47 in the pcDNA3 control group ($p < 0.01$) (fig. 8A). In mice bearing CT-26 tumors, significant suppression of lung metastasis was also achieved by EP-mediated IL-12 gene therapy (mean number of metastatic foci 7 ± 2 vs. 34 ± 11 for the pcDNA3 control group, $p < 0.05$) (fig. 8B). In addition, the sizes of the existing tumor nodules in the pIL-12-treated group were much smaller than those of the pcDNA3 control group, indicating that the growth of metastatic tumors was suppressed by IL-12 electro gene treatment. These results demonstrate that intramuscular electrotransfer of pIL-12 is an effective therapy against both subcutaneous and metastatic tumors originating from different organs.

Discussion

IL-12, delivered as a recombinant protein or by viral or nonviral vectors, demonstrates potent antitumor effects. However, these various IL-12 treatment approaches each has some major disadvantages. In the present study, we used in vivo EP to deliver plasmid pIL-12 into muscle tissues, and found that this method displayed superior antitumor effects compared to plasmid DNA delivered by direct muscle injection or by gene gun-mediated gene transfer. A single treatment of the IL-12 gene by in vivo EP cured animals bearing several subcutaneously implanted murine tumors and inhibited dissemination and growth of metastatic tumors. Intramuscular electrotransfer of pIL-12 even significantly prolonged the survival time of animals with advanced malignant diseases (within a week of death).

Direct injection of plasmid DNA into muscle holds promise as a convenient means of achieving gene transfer in vivo. Muscle fiber has a long life span and can be transduced by plasmid DNA [50, 51]. However, the relatively low expression levels of the transferred gene limit the application of this approach. In vivo EP provides a solution to overcome this limitation. It was recently demonstrated that intramuscular injection of plasmid DNA followed by EP with low voltage (200 V/cm) and a long pulse duration (50 ms) dramatically increased reporter gene expression in muscle tissue [1]. Using this approach, Rizzuto et al. [42] demonstrated that a therapeutic level of erythropoietin could be achieved to correct anemia due to chronic renal failure in a rat model. Aihara and Miyazaki [1] also reported that EP treatment of mouse muscle improved the uptake of a plasmid DNA and produced up to a 100-fold increase in circulating interleukin-5 compared with simple plasmid DNA injection. In agreement with the above findings, we found in the present study that electrotransfer of the IL-12 gene into mouse muscles resulted in an approximately 80-fold increase in transgene expression (fig. 2A). Significant serum IL-12 levels were detected 1 day after gene transfer; IL-12 concentrations peaked at day 5, gradually decreased to approximately 10% of the maximum value by 11 days after EP, and remained at this low but sustained level for up to 60 days (fig. 1). Histologic analysis showed inflammatory infiltrates and some necrotic fibers in the treated muscle tissue (experimental observation, data not shown). Nevertheless, tissue damage caused by electrostimulation was transient. This gene delivery approach can also be successfully applied to large animals. Draghia-Akli et al. [15] reported that a single intramuscular injection of plasmid encoding porcine growth hormone-releasing hormone (GHRH) followed by EP in 3-week-old piglets elevated serum GHRH levels by 2- to 4-fold and elicited growth in pigs with no associated toxicity. These results clearly demonstrate that in vivo EP provides a potentially safe and efficient approach to enhance the efficiency of muscle-targeted gene transfer to induce continuous production of therapeutic levels of proteins.

One of the most important biological functions of IL-12 is its ability to induce production of IFN-γ from T and NK cells [8, 18]. Hence, it is important to evaluate the IFN-γ production in animals treated with pIL-12 by in vivo EP. On day 5 after treatment, pIL-12 at doses of 100, 50, 10, and 5 μg produced 28,170, 9,210, 1,110, and 220 pg/ml of serum IFN-γ (fig. 2B and data not shown), respectively. The lower dose of the plasmid (1 μg DNA) produced only a low titer (130 pg/ml) of IFN-γ at day 2 after pIL-12 treatment, with the titer decreasing to an undetectable level by day 5. Electrotransfer of the control pcDNA3 plasmid produced no detectable serum IFN-γ (fig. 2B). We also noted that serum levels of IFN-γ after pIL-12 treatment paralleled the kinetics of IL-12 expression (fig. 1). Taken together, these data show that electrotransfer of the IL-12 plasmid was effective in inducing IFN-γ, and that the IFN-γ titer was dependent upon the dose of pIL-12 administered.

We also tested the in vivo antitumor activity of EP-mediated IL-12 gene transfer. Our initial studies on mice bearing day-3 subcutaneously implanted 38C13 B-cell lymphoma showed that intramuscular electrotransfer of 100 μg of pIL-12 induced a systemic antitumor effect and significantly increased the survival time of the animals (fig. 3). In fact, the majority (66%) of treated animals survived for more than 4 months and were apparently free of residual or dormant tumor cells, as demonstrated by in vivo culture and fluorescence-activated cell sorting analysis of spleen and lymph node cells (data not shown). Delivery of pIL-12 by in vivo EP was also effective in suppressing the growth of 2 other murine tumors, CT-26 colon adenocarcinoma and B16F1 melanoma (fig. 7). In addition, pIL-12/EP treatment also markedly inhibited the dissemination and growth of experimental lung metastases induced by intravenous injection of CT-26 or B16F1 cells (fig. 8). The mean number of metastatic foci was reduced by 87% in the B16F1 model and by 80% in the CT-26 model. Moreover, the sizes of the existing tumor nodules in the pIL-12/EP-treated group were much smaller than those in the pcDNA3 control group. These results demonstrate that intramuscular electrotransfer of the IL-12 gene was a potent antitumor therapy against both subcutaneous and metastatic tumors.

Even more-impressive results of the IL-12 electro gene therapy came from studies on treating large established 38C13 tumors, which are resistant to most immunotherapeutic regimens [7]. In the day-7-treated group, complete tumor regression was observed in most cases, and most animals (80%) survived for more than 80 days. Significant antitumor effects were also demonstrated in animals bearing very large tumors (approximately 1,000 mm^3) with systemic metastases (the day 14-treated group). Animals in this group usually died within a week without treatment. We found that a single treatment of these late-stage animals with pIL-12 by in vivo EP resulted in regression of 50% of the large subcutaneous tumors and significantly prolonged the lifespan of these animals (mean survival time 42 ± 18 vs. 25 ± 1 days for the pcDNA3 control group) (fig. 5C). Another intriguing finding of our EP-mediated IL-12 gene therapy was that this treatment consistently achieved better antitumor effects in the day 7-treated group than in the day 3-treated group. This result is consistent with several other studies which demonstrated that IL-12 treatment appears to be highly effective against large established tumors [30, 41, 56, 57].

We compared the antitumor activities of in vivo EP with other nonviral gene delivery techniques. A simple intramuscular injection of pIL-12 without electrostimulation had little effect on tumor growth (fig. 4D). Similarly, gene gun-delivery of pIL-12 produced no antitumor activity in the 38C13 tumor model even after 5 consecutive treatments with a large amount of DNA (fig. 4E and data not shown). Rakhmilevich et al. [41] previously reported that in vivo gene gun delivery of the IL-12 gene resulted in complete regression of established tumors in some tumor models (L5178Y, MethA, SA-1 and Renca), but was less effective in others (P815 and B16). Our preliminary data show that intramuscular electrotransfer of pIL-12 can cause complete regression of established P815 tumors. This result together with our results on 38C13 tumors clearly shows that in vivo EP represents a much more efficient delivery approach for cytokine gene therapy. The different therapeutic effects produced by these gene delivery methods were likely due to the amount of IFN-γ produced in vivo. In contrast to the significant titer of IFN-γ observed in EP-treated animals, delivery of the IL-12 gene by intramuscular injection or gene gun produced no detectable IFN-γ in sera.

The exact mechanism mediating the antitumor activity of IL-12 electro gene therapy is currently unknown. We speculate that the systemic IFN-γ stimulated by pIL-12 EP might play a major role in causing tumor eradication. Previous studies have shown that the antitumor activity of IL-12 is largely dependent on its ability to induce the expression of IFN-γ, which mediates tumor regression by stimulation of macrophages, upregulation of the expression of major histocompatibility complex proteins on tumor cells, induction of IFN-inducible protein 10, and inhibition of angiogenesis [13]. CD4+/CD8+ T cells and NK cells contribute most to IFN-γ production after IL-12 treatment. In this study, we observed that the antitumor effect of EP-transferred pIL-12 was dependent upon the amount of DNA injected. Lower doses of pIL-12 (1 and 10 μg of DNA) elicited much less IFN-γ and produced no antitumor activity (fig. 3). This result indicates that production of IFN-γ above a threshold concentration by IL-12 electro gene therapy is critical for its antitumor effect.

Another interesting observation of our study is that intramuscular electrotransfer of pIL-12 could induce long-term protection in the 38C13 model. Mice previously treated with pIL-12/EP and which remained tumor-free were partially resistant to rechallenge with 38C13 tumors at day 60 following the initial treatment. Our results show that 25% (4 of 16) of mice in the pIL-12/EP-cured group survived the rechallenge (fig. 6B), and the rest of the animals also showed significant prolongation of life compared with mice in an age-matched naive group (fig. 6A). This long-term protection could be due to induction of

38C13-specific immunity by the previous pIL-12 electro gene therapy. The idiotypic protein of the surface immunoglobulin of 38C13 B-cell lymphomas is tumor specific and can induce idiotype-specific antibodies [6] and T cell immune responses [23] which protect animals against 38C13 tumor challenge. However, we found no detectable anti-idiotype antibody in animals that had been cured of 38C13 tumors by IL-12 electro gene therapy before rechallenge (data not shown), indicating that the anti-idiotype antibody played no role in the observed long-term protection. Whether T cell immunity against the idiotypic protein or other undefined tumor antigens of 38C13 are involved in the long-term protection is not clear at this moment. Another explanation for the long-term protection in the pIL-12/EP-cured group could be the continued production of IL-12 by the previous IL-12 gene treatment. In fact, a low but sustained level of serum IL-12 was present at day 60 before the rechallenge (fig. 1). Whatever the mechanism, these results show that a single intramuscular delivery of the IL-12 plasmid by EP is able to induce long-term, systemic protection against residual or dormant tumor cells.

Although we found a significant antitumor effect of EP-mediated IL-12 gene therapy, toxicity problems associated with IL-12 treatment still remained. Electrotransfer of up to 100 μg of pIL-12 DNA resulted in no gross toxicity in adult mice, and all animals survived the therapy. However, we observed a toxic effect in young mice (less than 6 weeks old) treated with a high dose (100 μg) of pIL-12 as reflected by suboptimal weight gain and even mortality. Such IL-12-mediated toxicity has been previously reported in adult mice after repeated injections of high doses of recombinant IL-12 [38]. Therefore, additional modalities of the EP-mediated IL-12 gene transfer method are necessary to overcome toxicity problems. There are 2 possible solutions to this issue. First, the IL-12 gene may be directly electrotransferred into tumor cells to avoid systemic toxicity. In vivo EP has been used with success for gene transfer to melanomas [43], gliomas [36], and colon carcinomas [19]. Recent reports by Yamashita et al. [52] and Lohr et al. [27] demonstrated that electrotransfer of the IL-12 plasmid into tumor tissues indeed significantly inhibited tumor growth in vivo. The limitation of this approach is that it cannot treat micrometastatic diseases. Second, plasmids encoding immunocytokines may be delivered by intramuscular electrotransfer. Immunocytokines are fusion proteins consisting of a tumor-specific monoclonal antibody and a cytokine molecule[26]. Immunocytokines can specifically target tumor cells and direct the attached cytokines to the tumor site. This specific targeting ability of immunocytokines should reduce the systemic toxicity associated with EP-produced cytokines. In one study, an Ab-IL-12 fusion protein against Her-2/neu was constructed and was found to possess potent antitumor activity [40]. We believe that intramuscular electrotransfer of a plasmid encoding Ab-IL-12 could provide a much simpler way to produce a therapeutic level of this fusion protein.

In summary, we have shown that intramuscular electrotransfer of the IL-12 gene is effective in inhibiting the in vivo growth of established subcutaneous and metastatic tumors. This approach is simple, inexpensive and has little intrinsic toxicity. Application of in vivo EP for the transfer of cytokine genes may represent a novel therapeutic strategy for cancer treatment.

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